STUDYING TRYPTOPHAN FLUORESCENCE TO DETERMINE MEMBRANE BURIAL: ATOMIC DETAIL INSIGHT FROM UNBIASED MOLECULAR DYNAMICS SIMULATIONS

by

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An essay submitted to Johns Hopkins University in conformity with the requirements for the Master of Science and Engineering

Baltimore, Maryland

May, 2017

ABSTRACT

Membrane-protein interactions play an important role in a wide variety of biological processes. These interactions are fundamental, as the interactions between peptides and lipids are the driving force for the mechanisms of numerous membrane-mediated cellular processes. Tryptophan (Trp) fluorescence measurement is an analytical tool to measure protein and peptide interactions with lipid membranes. Particularly, it is used to determine the depth of membrane burial of peptides containing an indole side chain in protein structures. Indole groups are solvent-sensitive fluorophores, and therefore, the location of tryptophan residues in proteins can be observed via their emission spectra. While the degree of solvation of the Trp side chain in phospholipid membranes can be determined with high accuracy using the fluorescent spectra of successive lipid titrations, the atomic detail configurations of the buried states remain unknown. This study quantitatively compares the burial of Trp *in vitro* via fluorescence measurement and *in silico* via molecular dynamics (MD) simulations. Fluorescence measurements of acetylated Trp containing penta-peptides WLRLL showed binding in both simulations and fluorescence measurements. However, the pentapeptide WARAA showed no partitioning using fluorescence, while atomistic trajectories showed that the penta-peptide bind strongly to the interface of the model membrane, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). This suggests that there might be an error in MD simulations force fields, and further improvement especially of the water model may be needed to achieve a precisely and accurately capture the biological processes.

DEDICATION

This essay is dedicated to my loving husband and daughter, Tuan Mohd Faris and Tuan Naurah Fellah.

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INTRODUCTION

Protein-membrane interactions have been widely studied over the last few decades. This important topic attracts thousands of studies to explore how these interactions contribute towards essential biological functions in an organism. The research is not only looking at the fundamental process of particular interactions, but it also opens up new directions for medical purposes - such as novel therapeutic drug discovery for improving the lives of humans especially.

Clearly, the lipid and protein components of a biological membrane must have coevolved to permit membrane proteins to function in the environment provided by the lipid bilayer and inserted without any damage[1]. Useful knowledge about lipid–protein interactions has started to loom from high-resolution structural studies of membrane proteins, which sometimes include lipid molecules[1]. However, the structural and functional characterization of lipid-protein interactions in biological membranes has always been an enormous challenge to cell biology. Although many biochemical techniques exist for measuring protein-protein interactions, there has been a lack of tools for protein-lipid interactions.

Methods for peptide – lipid bilayer interaction study

Various biochemical and biophysical methods have been developed to study protein – lipid interactions. Among the methods are lipid overlay assay, sedimentation assay using lipid vesicles, fluorescence methods, surface plasmon resonance (SPR) analysis[2], and isothermal titration calorimetry (ITC). Each of these methods has its own advantages. However, there are also major limitations that need to be taken into account when deciding on an assay. Lipid overlay assay, for example, is an easy and simple method, but has low sensitivity, poor realibility and is unable to generate quatitative information[3]. Pelleting vesicles and protein lebelling inefficiency makes the the vesicle sedimentation

assay more tedious than the others. The SPR analysis methods is proven to be highly sensitive, no labelling is required, and provide useful kinetic information that is important for such studies. However, expensive instrumentation and mesurement under nonequilibrium conditions[2] may affect the intrepretation of the collected data, limiting the utility of this method.

ITC can also be used to investigate secondary processes accompanying peptide-membrane interactions, such as membrane permeabilization, peptide induced lipid phase transitions, peptide aggregation at the membrane surface and peptide conformational changes. Nevertheless, ITC is an excellent tool to study the entropic and enthalpic contributions to binding activity[4]. Calorimetric titrations, however, often require larger amounts of material and higher concentrations than comparable fluorescence experiments. These requirements can present particular problems if the protein under study is difficult to purify or has low solubility.

Various fluorescence techniques have been employed to monitor membrane-protein interaction. Techniques such as anisotropy and fluorescence correlation spectroscopy analyses however, has been hampered by their respective drawbacks and limitations.

Tryptophan fluorescence assay

So what do we measure in a fluorescence assay? The answer is the fluorescence intensity, *I* and the intensity can be measured as a function of wavelength, λ. There are over 3000 articles published in PubMed over the last 10 years that exploits the studies of tryptophan (Trp) fluorescence in protein-membrane interactions. Apart from fluorescence intensity, these studies also utilized the wavelength maximum (λ_{max}), fluorescence lifetime, anisotropy, and energy transfer to study interactions such as folding and unfolding; substrate binding; and exterior quencher accessibility[5].

	Absorption		Fluorescence	
	λ (nm)	**Absorptivity (ε)**	**λ (nm)**	**Quantum Yield**
Tryptophan	**280**	**5600**	**348**	**0.2**
Tyrosine	**274**	**1400**	**303**	**0.14**
Phenylalanine	**257**	**200**	**282**	**0.04**

Table 1. The absorption and fluorescence values for three amino acid with indole moieties. These three amino acid residues that are primarily responsible for the inherent fluorescence of proteins have distinct absorption and emission wavelength.

It is clearly shown that addition of a Trp to a peptide sequence will give an enormous advantages over the others in designing a peptide for potential drug development. The indole group of Trp is the more dominant source of UV absorbance at compared to Tyr in protein. Also, fluorescence of Tyr in native protein is often quenched, presumably by its interaction with the peptide chain or via energy transfer to Trp[6,7]. Whereas Tyr may be regarded as a relative simple fluorophore, the spectroscopic properties of Trp are complex, in particular, the high sensitivity to the local environment.

Due to the above reasons, tryptophan fluorescence is a powerful analytical tool to measure proteins and peptide interaction on the phospholipids membranes. It is very sensitive to the change of the polartiy in local environment[8]. The indole group of tryptophan residues in proteins is one such solvent-sensitive fluorophore, and the emission spectra of indole can reveal the location of tryptophan residues in proteins.

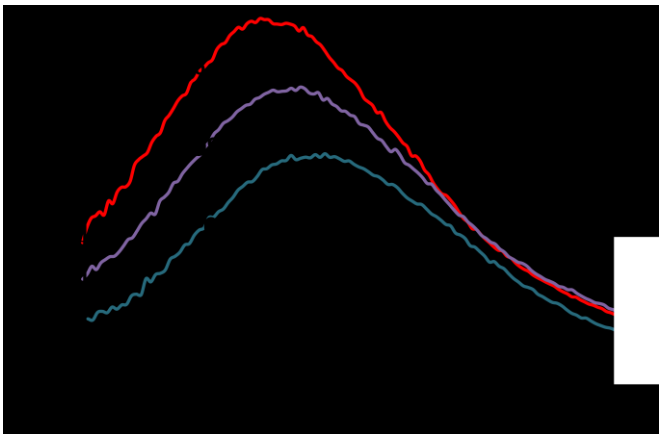

Figure 1. Illustrative example of bound and unbound state from Trp fluorescence assay. The increase in peak intensities and wavelength shifts indicate the partitioning of peptide to the bilayer, from 0 – 100 % bound.

As stated by Ladokhin (2000), the spectroscopically observed parameter should be a linear-response function; where the fractional change in the parameter should coincide with the fractional change of a particular molecular species, such as bound versus unbound or folded versus unfolded. With that, the Trp fluorescence of the bound or folded species is usually accompanied by a blue shift represented by red curve and increase in intensity as describe in Fig. 1. The spectrum for a free peptide in solution is represented by 0% bound and 100% bound when peptide is fully bound to the membrane[9].

Integration of experimental and computational approach to study the peptide-lipid interactions

The Trp fluorescecence assay seems straightforward and achievable. However, it does not provide any microscopic information of the peptide-lipid interactions. Various possible structural arrangements in the environment and the peptide itself may lead to a similar fluorescence signal [6]. As much as multiple bound states and poorly separated transitions; it is likely that the binding model selected for analysis will be too simple.

In fact, no single experimental technique can give a complete structural picture of the interaction, but rather a mix of different techniques is imperative[10]. The structural characterization of membrane active peptides in membranes is a harsh experimental challenge. Therefore, computational methods have been widely used for studying peptide membrane interactions primarily to obtain the atomistic view of the particular event. More mechanistic information can be attained with the implementation of molecular dynamic (MD) simulation. MD simulation is now a well established tool to compute protein–lipid-binding structures *in silico,* providing atomic-scale insight into the structure and dynamics of membranes that is not accessible experimentally[11].

Limitations from the experimental data clearly suggest the need for an atomic detail bilayer description. Determination of the partitioning properties of polypeptide segments into lipid bilayers are critical to understanding the insertion, assembly, and function of membrane proteins. Unfortunately, experiments aimed at directly measuring the transfer of peptides from water into bilayers have not so far been successful, primarily because sequences that are sufficiently hydrophobic to spontaneously insert without disrupting the membrane have a tendency to aggregate. Experimental artifacts in tryptophan fluorescence measurement can interfere with the method of determining the reversible equilibrium thermodynamics stability of proteins using titrations of chemical denaturants [9,12]. Computational methods in which the membrane is represented implicitly have been developed and applied to simulate folding and insertion of small hydrophobic peptides, providing a confirmation of the folded insertion pathway and rough estimates of the energetics involved.

Aim of the study

Studies conducted by Wimley and coworkers measured the membrane binding of penta-peptides with different 'guest'(X) side-chain located in the sequence Ac-WLXLL, which provided quantitative measurement of peptide-lipid interactions. However, these data do not provide us with atomistic details of such interactions. For example, the structures of the peptide throughout the partitioning process and also its orientation remain unknown. My studies incorporated MD simulations as well as Trp fluorescence assay to reveal the interaction of penta-peptides with membranes. This allows me to obtain full atomic insight into the partitioning process as well as quantitative partitioning data. I hope to find the unanimous stage where experimental binding assays agree with the simulation data. The study will give further insight into which elements need to be improved; experimental and/or computational methods or parameters, for a better understanding of how protein–lipid interactions drive biological systems.

MATERIALS & METHODS

Materials

Ace-WLRLL AND Ace-WARAA peptides

Peptides were purchased from WatsonBio Inc. (Houston, Tx). These peptides were chosen based on their solvation energy study by Wimley and colleagues[13]. The purity of the peptides was greater than 98%, and the peptide's identity was confirmed by mass spectrometry. The peptides were all monomeric in solutions in water under the conditions of the partitioning experiments as described in previous study[14]. The concentration of tryptophan and tryptophan-containing peptides were determined using extinction coefficient of 5600 $M^{-1}cm^{-1}$ at 280 nm using a NanoDrop 2000c Thermo Fischer Scientific (Wilmington, DE).

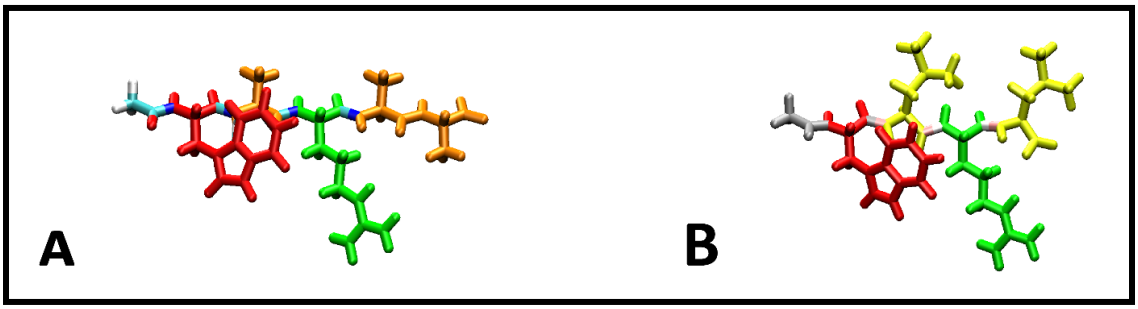

Figure 2. Shows the structure of two penta-peptides that were used in this study. Both peptides contain amino acid Tryptophan (residue 1) and Arginine (residue 3). The N-terminal is acetylated and T-terminal is both in free form (-COOH). A) Poly-Alanine peptide, Ace-WARAA and B) Poly – Leucine peptide, Ace-WLRLL.

Large Unilamellar Vesicles (LUV) preparation

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were obtained from Avanti Polar Lipids (Alabaster, AL). LUVs are prepared by POPC extrusion using an Avanti extruder through 100 nm membranes. Samples were prepared by mixing peptide and LUV solutions with specific peptide to lipid ratios. The peptides were prepared at concentration of 20 μM, and the vesicles were prepared in the range of 0.02 – 4 mM.

Tryptophan Fluorescence measurement

Membrane partitioning was assessed at pH 7 using tryptophan fluorescence measurement by microplate reader BioTek Synergy 2 (Winooski, VT). Peptide and lipid solutions were prepared at pH 7 at total volume of 100 μL with peptide to lipid ratio ranges from 0.005 -1. Excitation light was fixed at 270 nm with opening slit at 9 nm, and emission light was collected from 300 to 450 nm at width slit 20 nm. The 96-well UV-Star® microplate by Greiner Bio-One (Kremsunster, Austria) were used that has the expanded optical window down to 230 nm. In addition to the interest peptides, we also performed titration with the vesicles within similar ranges of L- Tryptophan (Sigma – Aldrich, MO) at concentration of 20 μM as the control.

Molecular Dynamics (MD) Simulations

An extended peptide was setup in bulk water with the center of mass 15 Å from the top and bottom of the surface of a pre-equilibrated 42 POPC lipid bilayer. Na and Cl ions at concentration of 100 mM were added to the system with no biasing potentials. The system was neutralized by additional Cl ions. The system was equilibrated for 10 ns, and we applied position restraints to the peptides. The molecular dynamics simulations were performed with GROMACS 5.0.5 and Hippo BETA simulation packages (http://www.biowerkzeug.com). For the force field, OPLS united atom lipid parameters for POPC were taken from Ulmschneider and colleagues[15] and paired with the TIP3P water model[16]. Electrostatic interactions were computed using PME, and a cutoff of 10 Å was used for van der Waals interactions. Bonds involving hydrogen atoms were constrained using LINCS. The integration time-step was 2 fs, and neighbor lists were updated every 5 steps. All simulations were performed in the NPT ensemble, without any restraints or biasing potentials. Water and the protein were each coupled separately to a heat bath with a time constant τ_T = 0.5 ps using velocity rescale temperature coupling. Atmospheric pressure of 1 bar was maintained [17] using weak semi-isotropic pressure coupling with compressibility $\kappa_z = \kappa_{xy} = 4.6\times10^{-5} bar^{-1}$ and time constant τ_P = 1 ps.

At the end of simulations, the energy components were extracted from the energy files generated by the program and analyzed to verify the stabilization of the system. Later, several analyses were conducted using programs built within GROMACS, and results were visualized and elaborated with the aid of the freely available program Grace (http://plasma-gate.weizmann.ac.il/Grace) and VMD (http://www.ks.uiuc.edu/Research/vmd)

RESULTS & DISCUSSION

Peptides solubility

I purposely choose this penta-peptide because, from the original design, this penta-peptide sequence does not form secondary structure. However, one major drawback of these peptides is that they have very low predicted solubility. Surprisingly, both peptides were easily solubilized in MiliQ water, therefore reducing the potential interference of organic solvent into our fluorescence measurement.

Trp fluorescence measurement for peptide – vesicle binding assay

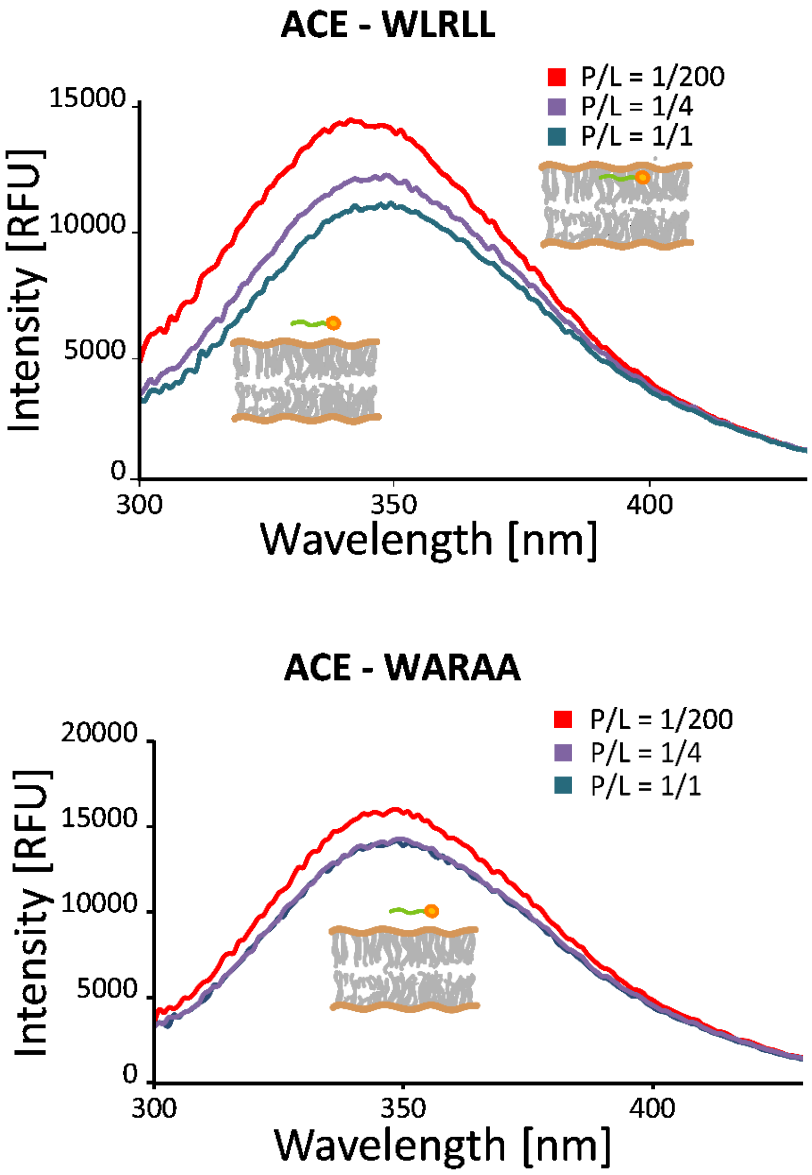

Figure 3. Comparison of Tryptophan fluorescence titration for Ace – WLRLL, and Ace - WARAA. The binding activity of peptide to lipid bilayer can be seen from the peak shift to the left and increase in intensity. Both peptides were prepared at 20 µM concentration.

Figure 3 compare the results of binding assay for both penta-peptides. As described above, the partitioning of a peptide into the bilayer can be seen from the wavelength shift

and also the increase in intensity. From the above figures, it clearly shown that upon titration with lipids the peak shifted and increased in intensity for Ace–WLRLL, which indicates the partitioning of the peptide into the bilayer. However, for peptide Ace-WARAA, I did not observe any of these events, which is very surprising. Both of these peptides have a net charge of 0, which eliminates any bias due to peptide–lipid charge interactions. The position and intensity maxima of the two measurements were found to be identical to each other in conditions without vesicles and very similar to free tryptophan in solution. I concluded, therefore, that the peptides probably did not aggregate in these concentration ranges which are approximately the same concentrations as were used in the partitioning experiments.

Although microplate reader was used for the measurement, all suggestions were followed to correct and reduce the effects of light scattering on tryptophan spectrofluorometry, as described by Ladokhin and coworkers[9], photolability remains as one of the drawbacks in fluorescence measurement using fluorescence molecules. This event results in irreversible degradation and photobleaching. I suspected this might be the cause of this scenario. The rate of photobleaching contributed by several factors, such as the fluorophore environment and the excitation wavelength [18].

Molecular Dynamics (MD) Simulation of Ace -WARAA

The structure of the penta-peptides was confirmed with built–in secondary structure analysis and Ramachandran available as built–in analysis in VMD.

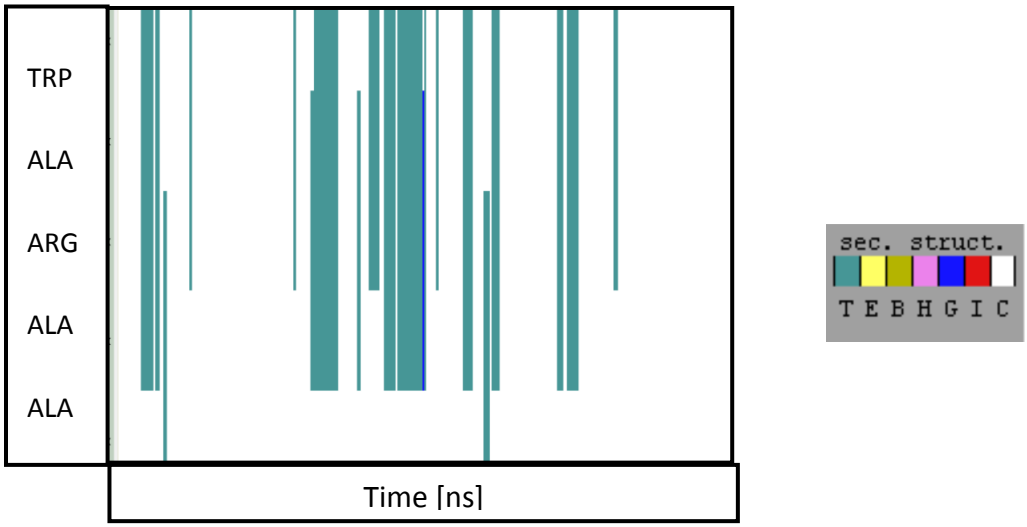

Figure 4. Secondary structure analysis of peptide Ace – WARAA. No secondary structure was observed from this analysis. Analysis was done using VMD built – in secondary structure analysis tools (http://www.ks.uiuc.edu/Research/vmd/).

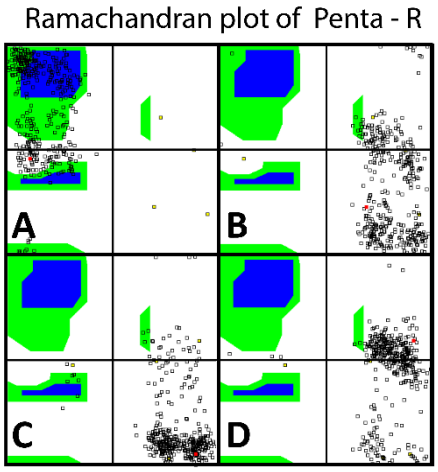

Figure 5. Ramachandran plot of the penta -R. The distribution of all residues show that this peptide does not form alpha-helical and beta-sheet structure. A) The trajectory of the residue 2 in the peptide sequence, Trp B) the trajectory of residue 3, Ala C) the trajectory of residue 4, Arg D) the trajectory of residue 5, Ala in Ramachandran space over times.

The simulation of Ace–WARAA was carried out for one microseconds. To my surprise, the trajectory of the simulations indicated that the peptide binds very strongly to the lipid bilayer. This observation contradicts with my findings from the fluorescence measurement data that suggested no binding activity. As indicated earlier, no secondary structure was observed throughout the simulations.

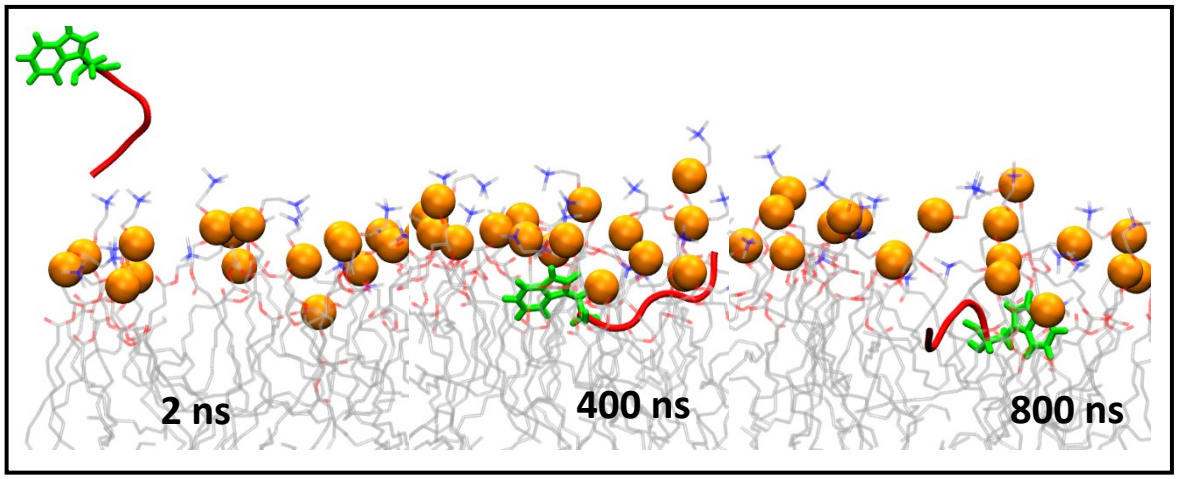

Figure 6. The snapshot of Ace -WARAA simulation in POPC bilayer. The peptide strongly binds to the bilayer interface without forming secondary structure.

For Ace-WLRLL and Ace-WLILL single pentapeptides were placed in ionic solution above a POPC lipid bilayer and allowed to freely fold and partition into the lipid. Both peptides rapidly absorbed to the membrane surface and stayed fully bound for the remainder of the 10 ns simulations. As expected, the peptides remained unstructured, alternating between extended and random coiled conformations. Both peptides were aligned nearly parallel to the bilayer surface, as deduce by fits through the peptide backbone (Figure 7, tilt)

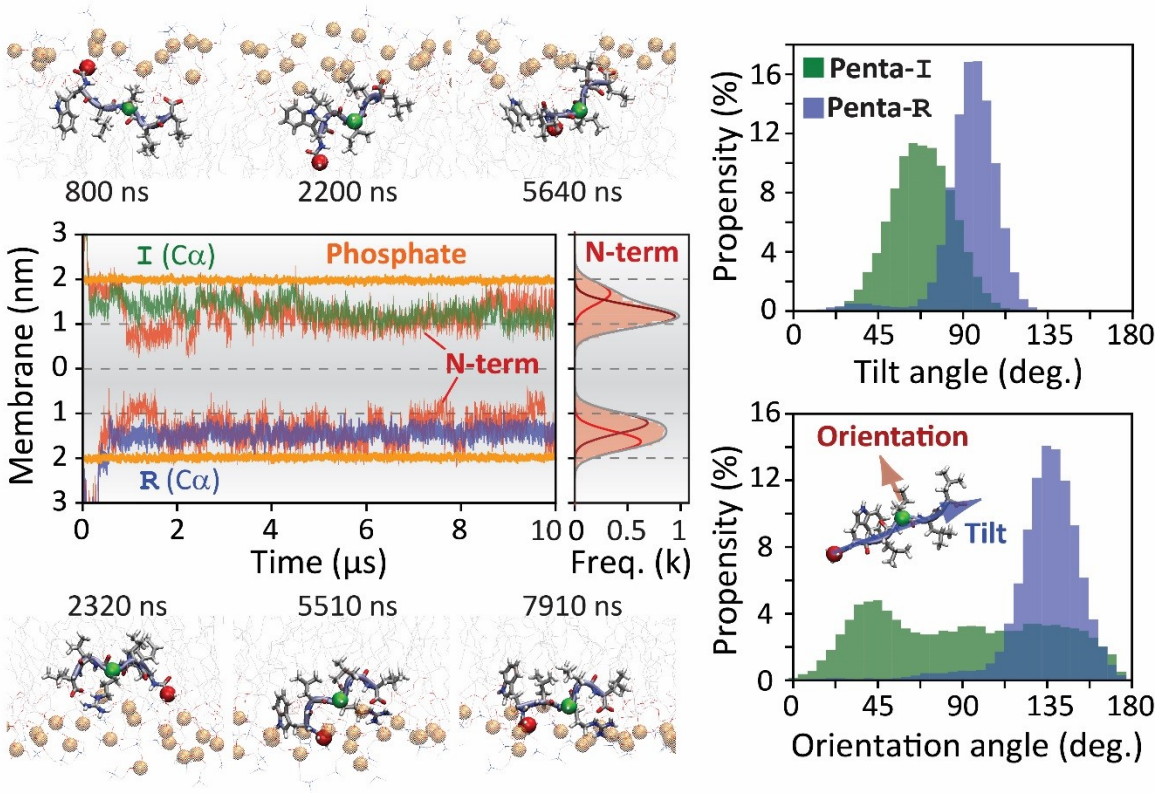

Figure 7. MD Simulations reveal multiple conformational sub-populations at equilibrium. Long-timescale unbiased equilibrium MD simulations show the peptides partitioning stably into POPC bilayers, roughly at the height of the glycerol-carbonyl linker. Position versus time traces and positional histograms showing side chain positions as well as the N-termini (N-term, red) are displayed. Both peptides exhibit multiple sub-populations in position space. Histograms of the tilt angle of the peptide backbone as well as the angular orientation of the guest sidechain indicate that penta-R has a preference for a defined tilt and orientation, whereas penta-I is more diverse, especially in orientation angle. Angles are defined from the membrane center outward: 0 deg towards the core, 180 deg out of the membrane. Insets: three snapshots of each peptide during the simulation (penta-I, top; penta-R, bottom), water has been removed for clarity. N-term and sidechain Ca are drawn as red and green balls, respectively.

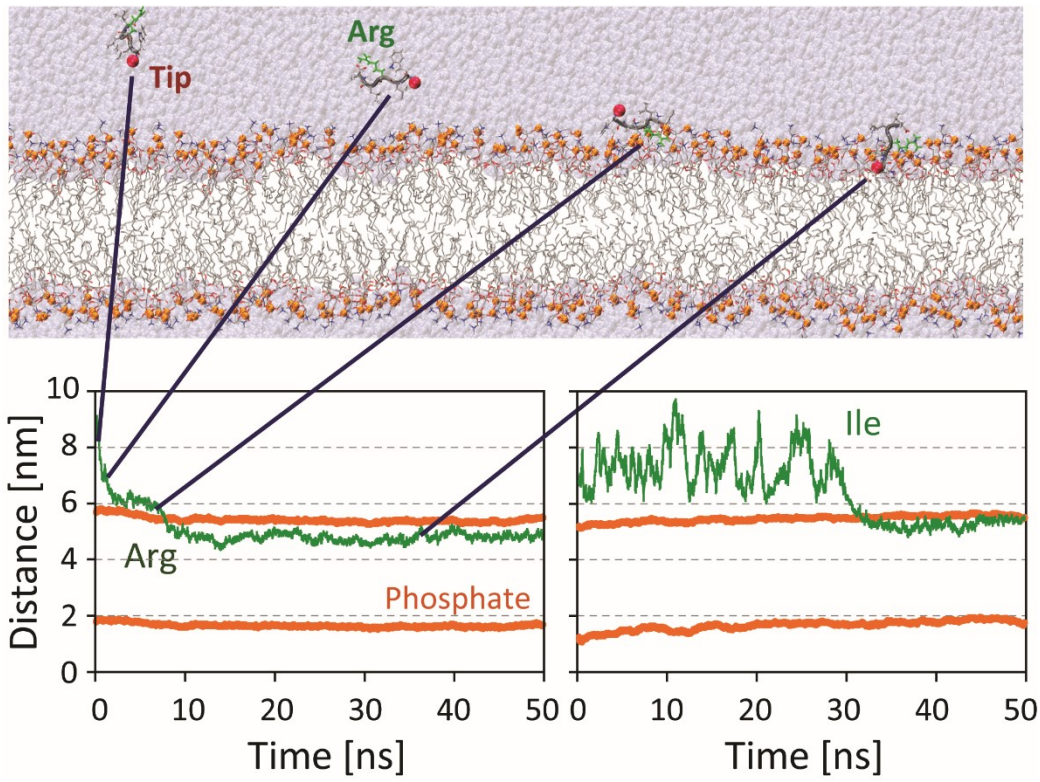

Figure 8. Unbiased partitioning simulation of the W-L-R-L-L and W-L-I-L-L peptides. The results demonstrate complex trajectories associated with the partitioning process that take place on the 10 ns timescale. Lengthening the simulations to 10 μs each found that peptide binding is irreversible, at least on the time scale of the simulation.

Presence of aqueous phase aggregation will compromise any effort to determine the energetics of peptide-membrane interactions, especially for longer hydrophobic sequence[19]. Consequently, knowledge of the aggregation state of hydrophobic peptides in water is of the utmost importance in the analysis of partitioning energetics. Although Trp fluorescence measurement is very sensitive to aggregation - the peak shift to the shorter wavelengths indicating this event; further analysis on the peptides might help reveal some of the information that we need. Analysis such as dynamics and steady state light scattering, fluorescence quenching, and fluorescence anisotropy[19] may shed some light on the event that is happening here.

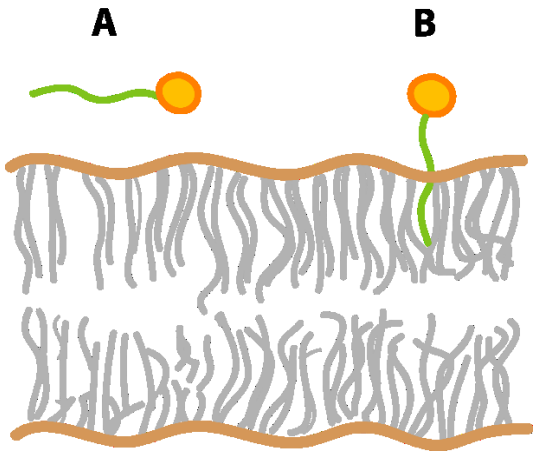

Figure 9. Possible Tryptophan position inside the lipid bilayer. A) Tryptophan in solution B) The indole side chain stays in solution, while the rest of the peptide embedded in the bilayer.

The position of the Trp may differ from the what can we interpreted from the measured fluorescence assay. Fig. 8 explains this event, where Trp in position B may be considered as unbound state although the rest of the peptide bound into the lipid bilayer.

Implementation of distribution analysis (DA) methodology could generate better quantitative information on the membrane penetration, and may be helpful to overcome the above problem. To do this, depth dependent fluorescence quenching experiment can be conducted[11]. It provides not only the average position of the fluorophore in the

bilayer, but also other important structural characteristics as well, such as transverse heterogeneity (via the width of the distribution) and the exposure of the probe to lipids[11].

However, it is unlikely for the experimental setup to have an error. This method is established and has been used for the past decades. It may turn out that the MD simulation force fields are not balanced. From the *in silico* results, it appears that the peptide-water and peptide–lipid interactions appear not to be balanced. Also, the complex physiochemical properties of fluid phase lipid bilayers, particularly in the interfaces, have proved difficult to capture with implicit solvation models[20] and potentially serious deficiency of simulations, which do not explicitly model all hydrogen atoms. From the findings, we suggest that there is a need to incorporate different water model, for example, TIP4P to perform simulations. The dataset that will be obtained from this improvement will allow a quantitatively precise dataset for force field calibration in the future.

CONCLUSIONS

Reliable methodologies that characterize the interactions of biomolecules offer tremendous value in advancing our systematic understanding of biological systems. Measuring the thermodynamic stability of membrane proteins folded in vesicles is approachable by monitoring the tryptophan fluorescence emission by the proteins, however, may need to be coupled by further experiments using different assays such as ITC to get a thorough understanding on the binding activity. Computational approaches, via MD simulation, have the advantage of providing an atomistic view of such events, however, these need further calibration on the force fields to mimic the 'real' biophysical processes. The integration of both approaches may allows researchers to reevaluate the measurement and analysis of tryptophan fluorescence in membranes properly. I hope that my findings and suggestions provided by MD simulations may corroborate experimental findings in the future.

ACKNOWLEDGEMENTS

I would like to express my thanks to my advisor, Dr. Martin Ulmschneider, and lab members, for giving continuous supports and encouragement throughout this period. I also would like to extend my thanks to my sponsors, Ministry of Higher Education Malaysia (MOHE) and my employer, Universiti Teknologi MARA Malaysia for giving me opportunity to be here in Johns Hopkins University.

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BIOGRAPHICAL STATEMENT

Zafirah Liyana Abdullah was born in Kuantan, Pahang, Malaysia in 1985. She received her B. Sc. with Edu. Majoring in Biology from University of Malaya, Malaysia and M. Pharm Sc. (Pharm. Tech.) from International Islamic University of Malaysia. She received a fellowship from Ministry of Higher Education Malaysia to pursue her study in Johns Hopkins University and currently works under Dr. Martin Ulmschneider studying computational biomaterials.